Structural characterization of glucosylated lactose derivatives synthesized by the Lactobacillus reuteri GtfA and Gtf180 glucansucrase enzymes

Pham, Hien T T; Dijkhuizen, Lubbert; van Leeuwen, Sander S

Published in:
Carbohydrate Research

DOI:
10.1016/j.carres.2017.07.002

Publication date:
2017

Carbohydrate Research 449 (2017) 59–64

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Structural characterization of glucosylated lactose derivatives synthesized by the *Lactobacillus reuteri* GtfA and Gtf180 glucansucrase enzymes

Hien T.T. Pham, Lubbert Dijkhuizen*, Sander S. van Leeuwen

Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Nijenborgh 7, 9747 AG Groningen, The Netherlands

ARTICLE INFO

Article history:
Received 3 April 2017
Received in revised form
22 June 2017
Accepted 6 July 2017
Available online 8 July 2017

Keywords:
Glucansucrase Gtf180-ΔN
Glucansucrase GtfA-ΔN
Trans-glycosylation
Lactose
^{1}H NMR spectroscopy

ABSTRACT

Glucansucrase enzymes from lactic acid bacteria are receiving strong interest because of their wide range of gluco-oligosaccharide and polysaccharide products from sucrose, some of which have prebiotic potential. Glucansucrases GtfA and Gtf180 from *Lactobacillus reuteri* strains are known to convert sucrose into α-glucans with different types of linkages, but also to use other molecules as acceptor substrates. Here we report that incubation of (N-terminally truncated versions of) these enzymes with lactose plus sucrose resulted in synthesis of at least 5 glucosylated lactose products of a degree of polymerization (DP) of 3–4. Only glucansucrase Gtf180-ΔN also produced larger lactose-based oligosaccharides (up to DP9). Structural characterization of the glucosylated lactose products DP3-4 revealed glycosidic bonds other than (α1→4)/(α1→6) typical for GtfA-ΔN and (α1→3)/(α1→6) typical for Gtf180-ΔN: Both GtfA-ΔN and Gtf180-ΔN now introduced a glucosyl residue (α1→3)- or (α1→4)-linked to the non-reducing galactose unit of lactose. Both enzymes also were able to introduce a glucosyl residue (α1→2)-linked to the reducing glucose unit of lactose. These lactose derived oligosaccharides potentially are interesting prebiotic compounds.

1. Introduction

Glucansucrase enzymes (Gtfs) of glycoside hydrolase family 70 (GH70) are extracellular enzymes that only have been identified in lactic acid bacteria (LAB) [1]. They catalyze three types of reactions, depending on the nature of the acceptor substrate: hydrolysis when water is used as acceptor substrate, polymerization when the growing glucan chain is used as acceptor, and transglycosylation when other compounds including oligosaccharides are used as acceptor [2]. The currently known diversity of glucansucrases is capable of synthesizing α-glucans with all the possible glycosidic linkage types [(α1→2), (α1→3), (α1→4) and (α1→6)]. They are classified into dextran-, mutan-, reuteran-, and alternansucrases based on the (dominant) linkage type(s) in their products [2–5]. The catalytic mechanism of Gtfs is similar to that of the family GH13 enzymes, namely an α-retaining double displacement reaction [2]. The reaction starts with the cleavage of sucrose, resulting in the formation of a covalent β-glucosyl-enzyme intermediate. This is followed by transfer of the glucosyl moiety to an acceptor substrate with retention of the α-anomeric configuration. In case of acceptor reactions, the orientation of the bound acceptor substrate towards the reaction center determines the type of linkages formed in the transglycosylation products [2]. Gtfs are able to transfer glucose to a wide variety of acceptors, either non-glycan compounds or oligosaccharide compounds, mostly disaccharides or disaccharide derivatives [4,5]. Maltose is a highly suitable acceptor substrate for Gtfs producing various products such as panose or other isomalto-oligosaccharides, while fructose is not a preferred acceptor for Gtfs [6]. Use of lactose as acceptor substrate has been previously studied for the dextransucrases from *Leuconostoc mesenteroides* and *Weissella confusa*, and the only transfer product that has been structurally identified is 2-α-D-glucopyranosyl-lactose [7,8]. The low cost of sucrose and lactose, combined with the broad acceptor substrate acceptance of glucansucrase enzymes, makes them useful tools in the synthesis of novel and potentially prebiotic oligosaccharides. This study explored the ability of glucansucrase enzymes Gtf180-ΔN and GtfA-ΔN from *L. reuteri* strains 180 and 121, respectively, to decorate lactose as acceptor substrate, using sucrose as donor

* Corresponding author.
E-mail address: L.Dijkhuizen@rug.nl (L. Dijkhuizen).

http://dx.doi.org/10.1016/j.carres.2017.07.002

substrate. While Gtf180-ΔN of *L. reuteri* 180 converts sucrose into a dextran with 69% (α1→6) linkages and 31% (α1→3) linkages [9], GtfA-ΔN catalyzes the synthesis of a reuteran consisting of 58% (α1→4) linkages and 42% (α1→6) linkages [10]. The transfer products synthesized by these two glucansucrases were structurally analyzed by high-pH anion-exchange chromatography (HPAEC), matrix-assisted laser-desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and 1D/2D $^{1}H/^{13}C$ nuclear magnetic resonance (NMR) spectroscopy (TOCSY, HSQC, ROESY). A total of five main structures were observed (DP3 and DP4) for both enzymes. Only in case of Gtf180-ΔN also longer oligosaccharides were observed.

2. Results

2.1. Transglucosylation of lactose

Initial reactions were performed with sucrose and lactose concentrations of 0.5 M (ratio of 1:1), at 37 °C and pH 4.7 during 24 h, which is the catalytic optimum of the Gtf180-ΔN and GtfA-ΔN enzymes for α-glucan synthesis from sucrose [1,2]. Blank reactions used only sucrose as both acceptor and donor substrate, mostly resulting in α-glucan synthesis. The HPAEC-PAD profiles of the oligosaccharide fractions of reactions with only sucrose (Fig. 1, line a) showed only a few minor peaks (reflecting that mostly polymerization occurred), besides clear peaks for glucose and fructose. The profiles of the oligosaccharide fractions of incubations with sucrose plus lactose of GtfA-ΔN (Fig. 1, line b) and Gtf180-ΔN (Fig. 1, line c) showed similar profiles, with five significant novel peaks F1-F5, besides minor peaks eluting later which are expected to be higher DP oligosaccharides with lactose (DP5—DP9). .

2.2. Structural analysis of transglycosylation products

Five major glucosylation products corresponding to peaks F1-F5 (Fig. 1) were isolated from the incubation mixture of Gtf180-ΔN for structural analysis by MALDI-TOF-MS and 1D/2D ^{1}H and ^{13}C NMR spectroscopy. The purity and retention time of each fraction was confirmed by reinjection on an analytical CarboPac PA-1 (4 × 250 mm) column. The fragment size distribution of each fraction was determined by MALDI-TOF MS. The data showed that three major products corresponded to trisaccharides, as evidenced by a pseudo-molecular sodium adduct ion at *m/z* 527 (F1-F3) and two products were tetrasaccharides, as evidenced by a pseudo-molecular sodium adduct ion at *m/z* 689 (F4 and F5) (Fig. S1). Each product fraction was analyzed by 1D ^{1}H NMR, as well as 2D ^{1}H-^{1}H and ^{13}C-^{1}H NMR spectroscopy.

2.2.1. Mono-glucosylated lactose compounds

2.2.1.1. Fraction F1. Trisaccharide F1 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The 1D ^{1}H NMR spectrum of F1 displayed four anomeric ^{1}H signals at δ 5.225 (**Aα** H-1, $^{3}J_{1,2}$ 3.79 *Hz*), 4.667 (**Aβ** H-1, $^{3}J_{1,2}$ 8.28 *Hz*), 4.510 (**B** H-1, $^{3}J_{1,2}$ 8.03 *Hz*) and 4.914 (**C** H-1 $^{3}J_{1,2}$ 4.49 *Hz*) (Fig. S2). All the ^{1}H and ^{13}C chemical shifts of these three residues were assigned by 2D ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC spectra (Table 2). The data showed that resonances of non-anomeric protons of glucosyl residue **Aα** and **Aβ** were not shifted compared to those values of the glucosyl residue observed in lactose (Table 2). Residue **B**, however, showed significant downfield shifts for H-3 and H-4 at δ 3.75 (Δδ + 0.09 ppm) and 4.027 (Δδ + 0.10 ppm), respectively. The position of residue **B** C-4 at δ 78.4 ppm (Δδ + 8.4 ppm), is indicative for substitution on the O4 of residue **B**. This is further supported by the 2D ROESY inter-residual cross-peak between **C** H-1 and **B** H-4 (Fig. S2). Residue **C** showed a ^{1}H and ^{13}C chemical shift pattern fitting a terminal residue [11]. Combining all data, the structure of trisaccharide compound F1 is determined to be α-D-Glc*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-D-Glc*p* (Table 1).

2.2.1.2. Fraction F2. Trisaccharide F2 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The ^{1}H anomeric signals of fraction F2 were revealed by 500-MHz 1D ^{1}H NMR spectrum as following δ 5.433 (**Aα** H-1, $^{3}J_{1,2}$ 3.49 *Hz*), δ 4.816 (**Aβ** H-1, $^{3}J_{1,2}$ 8.00 *Hz*), δ 4.465 (**B** H-1, $^{3}J_{1,2}$ 7.43 *Hz*), δ 5.355 (**D**$^{\alpha}$ H-1, $^{3}J_{1,2}$ 3.82 *Hz*) and 5.094 (**D**$^{\beta}$ H-1, $^{3}J_{1,2}$ 3.75 *Hz*) (Fig. S3). Using 2D ^{1}H-^{1}H TOCSY and 2D ^{1}H-^{13}C HSQC, all non-anomeric proton resonances were assigned (Table 2). The anomeric resonance value at δ 5.433 ppm of **Aα** H-1 is the structural-

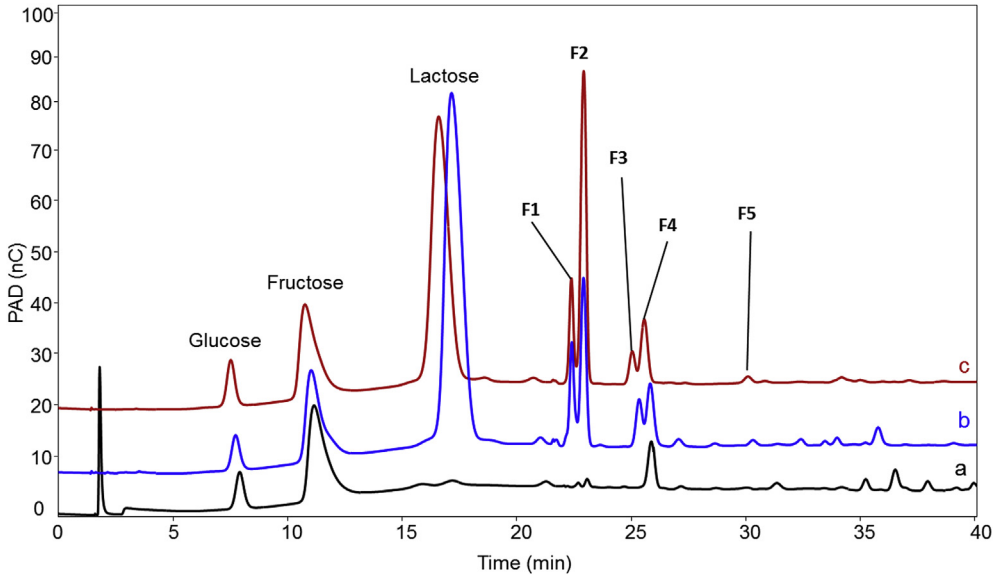


Fig. 1. HPAEC-PAD chromatograms of the reaction product mixtures obtained with 3 U mL^{-1} (a) GtfA-ΔN with 0.5 M sucrose; (b) GtfA-ΔN with 0.5 M sucrose and 0.5 M lactose; and (c) Gtf180-ΔN with 0.5 M sucrose and 0.5 M lactose. Reaction conditions: 24 h incubations at 37 °C and pH 4.7.

Table 1
Structures of the characterized oligosaccharide products F1-F5 of Gtf180-ΔN and GtfA-ΔN obtained with lactose and sucrose.

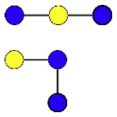
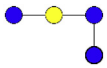
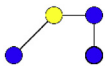
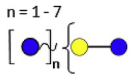
Nr.	Structures	Graphical presentation	Catalytic activity by
F1	α-D-Glc*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-D-Glc*p* **C**1 → 4**B**1 → 4**A**		 D-Glc*p*
F2	α-D-Glc*p*-(1 → 2)-[β-D-Gal*p*-(1 → 4)-]D-Glc*p* **D**1 → 2[**B**1 → 4]**A**		
F3	α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p* **C**1 → 3**B**1 → 4**A**		Gtf180-ΔN and GtfA-ΔN
F4	α-D-Glc*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p* **C**1 → 4**B**1 → 4[**D**1 → 2]**A**		 D-Gal*p*
F5	α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p* **C**1 → 3**B**1 → 4[**D**1 → 2]**A**		
Elongated glucosyl lactose derivatives			Gtf180-ΔN

Table 2
^{1}H and ^{13}C chemical shifts of the glucosylated lactose derivatives, measured at 300 K in D_2O. Chemical shifts that are key in the structural determination are underlined.

	Lac		F1		F2		F3		F4		F5	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
Aα1	5.222	92.8	5.225	92.7	5.433	90.0	5.225	92.8	5.439	90.0	5.437	90.2
Aα2	3.58	72.4	3.57	72.4	3.68	80.0	3.57	72.4	3.68	79.9	3.69	79.4
Aα3	3.83	72.5	3.82	72.5	3.94	70.5	3.82	72.5	3.94	72.8	3.96	72.8
Aα4	3.66	79.8	3.66	79.5	3.72	79.9	3.66	79.8	3.73	79.5	3.72	78.6
Aα5	3.95	71.2	3.950	71.2	4.00	70.5	3.95	71.2	3.99	70.9	4.00	70.9
Aα6a	3.87	61.5	3.88	61.2	3.86	60.8	3.88	61.5	3.87	61.2	3.88	61.2
Aα6b	3.84		3.84		3.65		3.84		3.84		3.84	
Aβ1	4.662	96.9	4.667	97.1	4.816	96.7	4.667	96.9	4.818	97.1	4.823	97.0
Aβ2	3.287	75.0	3.276	74.8	3.414	80.0	3.276	75.0	3.408	79.2	3.416	79.3
Aβ3	3.63	75.4	3.63	75.5	3.72	75.8	3.63	75.4	3.73	74.1	3.72	74.0
Aβ4	3.66	79.8	3.66	79.5	3.70	79.9	3.66	79.8	3.69	79.5	3.70	78.6
Aβ5	3.60	75.8	3.60	76.1	3.60	76.4	3.60	75.8	3.59	75.8	3.60	75.8
Aβ6a	3.95	61.6	3.95	61.2	3.95	60.8	3.95	61.6	3.84	61.2	3.97	61.2
Aβ6b	3.80		3.80		3.80		3.80		3.974		3.80	
B1	4.447	104.4	4.510	104.0	4.465	103.6	4.525	103.9	4.523	104.4	4.525	103.9
B2	3.54	72.3	3.58	72.2	3.54	72.1	3.66	70.6	3.58	71.8	3.66	70.6
B3	3.66	73.7	3.75	72.8	3.66	73.2	3.76	78.6	3.76	72.8	3.76	78.6
B4	3.92	69.8	4.027	78.2	3.920	69.4	4.161	66.1	4.019	78.1	4.161	66.1
B5	3.72	76.4	3.78	76.1	3.96	75.9	3.71	76.0	3.67	76.4	3.71	76.0
B6a	3.80	62.2	3.80	62.2	3.77	61.7	3.80	62.2	3.83	61.5	3.80	62.2
B6b	3.75		3.74		3.70		3.75		3.78		3.75	
C1			4.914	101.1			5.103	96.7	4.908	101.0	5.103	96.7
C2			3.55	72.6			3.56	72.2	3.54	72.7	3.56	72.2
C3			3.75	73.9			3.80	79.8	3.75	73.9	3.80	79.8
C4			3.466	69.8			3.46	70.4	3.47	70.2	3.46	70.4
C5			4.145	72.9			3.96	72.8	4.151	72.8	3.96	72.8
C6a			3.80	61.2			3.84	61.2	3.82	61.2	3.84	61.2
C6b			3.75				3.78		3.74		3.78	
Dα1					5.094	97.1			5.097	97.4	5.095	97.5
Dα2					3.54	72.1			3.56	72.7	3.59	72.4
Dα3					3.80	73.6			3.80	73.6	3.80	73.8
Dα4					3.46	69.8			3.46	70.2	3.46	70.4
Dα5					3.98	72.3			3.97	72.9	3.98	72.8
Dα6a					3.88	60.8			3.81	61.2	3.88	61.2
Dα6b					3.80				3.77		3.80	
Dβ1					5.355	98.5			5.374	98.7	5.355	98.8
Dβ2					3.53	72.1			3.54	72.7	3.53	72.4
Dβ3					3.75	73.6			3.75	73.9	3.75	74.0
Dβ4					3.47	69.8			3.47	70.2	3.47	70.4
Dβ5					4.084	72.1			4.075	72.7	4.089	72.7
Dβ6a					3.92	60.8			3.80	61.2	3.92	61.2
Dβ6b					3.80				3.75		3.80	

reporter-group signal of the 2-substituted reducing α-D-Glc*p* unit [11]. Additionally, strong downfield shifts were detected for **Aα** H-2 at δ 3.68 (Δδ + 0.10 ppm); **Aα** C-2 at δ 80.0 (Δδ + 7.60 ppm), **Aβ** H-2 at δ 3.41 (Δδ + 0.13 ppm) and **Aβ** C-2 δ 80.0 (Δδ + 5.00 ppm), confirming the substitution at O-2 of this residue. In residue **D**, $\mathbf{D}^{\alpha}$ H-5 and $\mathbf{D}^{\beta}$ H-5 signals are shifted downfield to δ 4.084 (Δδ + 0.13) and δ 3.98 (Δδ + 0.38), respectively, compared to corresponding signals of the glucosyl residue of lactose, as an indicator for the α-D-Glc*p*-(1 → 2)- unit [11]. This is further supported by ROESY inter-residual cross-peak between $\mathbf{D}^{\beta}$ H-1 and **Aα** H-2/$\mathbf{D}^{\alpha}$ H-1 and **Aβ** H-2 (Fig. S3). Meanwhile the set of chemical shifts values of residue **B** remained the same as those measured for lactose [12], indicating that no further substitution occurred at this residue. Considering all data together, the structure of trisaccharide compound F2 is determined to be α-D-Glc*p*-(1 → 2)-[β-D-Gal*p*-(1 → 4)-]D-Glc*p* (Table 1).

2.2.1.3. Fraction F3. Trisaccharide F3 includes 3 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively) and **C** (transferred glucosyl residue from sucrose) (Table 1). The 1D ^{1}H NMR spectrum of fraction F3 found four anomeric signals at δ 5.225 (**Aα** H-1, $^{3}J_{1,2}$ 3.89 *Hz*), δ 4.667 (**Aβ** H-1, $^{3}J_{1,2}$ 7.98 *Hz*), δ 4.525 (**B** H-1, $^{3}J_{1,2}$ 7.88 *Hz*) and δ 5.103 (**C** H-1, $^{3}J_{1,2}$ 3.53 *Hz*) (Fig. S4). The non-anomeric proton resonances were assigned by using 2D ^{1}H-^{1}H TOCSY and 2D ^{1}H-^{13}C HSQC (Table 2). The data showed that the ^{1}H and ^{13}C NMR patterns of residue **A** match with those values of the glucosyl residue observed in lactose [12]. Strong downfield shifts of residue **B** were detected for H-3 at δ 3.76 (Δδ + 0.10 ppm) and C-3 at δ 78.6 (Δδ + 4.90 ppm), suggesting the occurrence of (→3)-D-Gal*p*. This is confirmed by the 2D ROESY inter-residual cross-peak between **C** H-1 and **B** H-3 (Fig. S4). This substitution strongly influenced the chemical shift values of neighboring residue **B** H-4 (δ 4.161; Δδ + 0.24 ppm). The chemical shift of **C** H-4 at δ 3.465 are indicative for the terminal residue [11]. Combining all data together, the structure of disaccharide compound F3 was determined to be α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-D-Glc*p* (Table 1).

2.2.2. Di-glucosylated lactose compounds

2.2.2.1. Fraction F4. Tetrasaccharide F4 includes 4 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively), **C** and **D** (transferred glucosyl residues from sucrose) (Table 1). All NMR chemical shifts of compound F4 were assigned by 1D ^{1}H NMR, 2D ^{1}H-^{1}H TOCSY NMR, 2D ^{1}H-^{13}C HSQC NMR. The 1D ^{1}H NMR spectrum of fraction F4 detected six anomeric signals at δ 5.439 (**Aα** H-1, $^{3}J_{1,2}$ 3.48 *Hz*), δ 4.818 (**Aβ** H-1, $^{3}J_{1,2}$ 7.90 *Hz*), δ 4.523 (**B** H-1, $^{3}J_{1,2}$ 7.73 *Hz*), δ 4.908 (**C** H-1, $^{3}J_{1,2}$ 7.53 *Hz*), δ 5.374 ($\mathbf{D}^{\alpha}$ H-1, $^{3}J_{1,2}$ 3.87 *Hz*) and δ 5.097 ($\mathbf{D}^{\beta}$ H-1, $^{3}J_{1,2}$ 3.63 *Hz*) (Fig. S5). The anomeric signal of **Aα** H-1 at δ 5.439 ppm is indicative for the occurrence of a 2-substituted glucosyl residue [11]. Moreover, the set of ^{1}H and ^{13}C chemical shifts of residue **A** matched very well with those values of this residue found in compound F2, reflecting the existence of an α-D-Glc*p*-(1 → 2)- unit. This is further reflected by 2D ROESY inter-residual cross-peaks $\mathbf{D}^{\beta}$ H-1/**Aβ** H-2 (Fig. S5). Residue **D** showed downfield shifts of $\mathbf{D}^{\alpha}$ H-5 and $\mathbf{D}^{\beta}$ H-5 to δ 4.075 (Δδ + 0.13) and δ 3.97 (Δδ + 0.37), respectively, indicating the terminal residue at the branching side [11]. The resonances of **B** H-4 and C-4 at δ 4.019 (Δδ + 0.10 ppm) and δ 78.1 (Δδ + 8.3 ppm), respectively, showed considerable downfield shifts compared to those observed in lactose, suggesting the 4-substitution of the galactosyl residue. The 2D ROESY inter-residual cross-peaks **C** H-1/**B** H-4 confirmed the 4-substition of residue **B**. The chemical shift pattern of residue **C** matched that of residue **C** in compound F1, fitting a terminal α-D-Glc*p*-(1 → 4)- residue, linked to the Gal-residue **B**. Taking all data together, the structure of tetrasaccharide compound F4 was determined to be α-D-Glc*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p*, as illustrated in Table 1.

2.2.2.2. Fraction F5. Tetrasaccharide F5 includes 4 hexose residues, namely **A**, **B** (glucosyl and galactosyl residues from original lactose, respectively), **C** and **D** (transferred glucosyl residues). All NMR chemical shifts of compound F5 were assigned by 1D ^{1}H NMR, 2D ^{1}H-^{1}H TOCSY NMR, 2D ^{1}H-^{13}C HSQC NMR. The 1D ^{1}H NMR spectrum of fraction F5 found six anomeric signals at δ 5.437 (**Aα** H-1, $^{3}J_{1,2}$ 3.48 *Hz*), δ 4.823 (**Aβ** H-1, $^{3}J_{1,2}$ 8.01 *Hz*), δ 4.525 (**B** H-1, $^{3}J_{1,2}$ 7.51 *Hz*), δ 5.103 (**C** H-1, $^{3}J_{1,2}$ 3.71 *Hz*), δ 5.355 ($\mathbf{D}^{\alpha}$ H-1, $^{3}J_{1,2}$ 3.82 *Hz*) and δ 5.095 ($\mathbf{D}^{\beta}$ H-1, $^{3}J_{1,2}$ 3.48 *Hz*) (Fig. S6). As discussed for compound F4, the anomeric signal of **Aα** H-1 at δ 5.437 ppm is indicative for the occurrence of a 2-substituted glucosyl residue [11]. The set of ^{1}H and ^{13}C chemical shifts of residue **A** matched very well with those values found for this residue in compound F2, indicating the existence of a (→2/4)-α-D-Glc*p*, which is further reflected by 2D ROESY NMR inter-residual cross-peaks $\mathbf{D}^{\beta}$ H-1/**Aβ** H-2 (Fig. S6). The resonances of residue **B** H-3 and C-3 have been shifted to δ 3.76 (Δδ + 0.10 ppm) and δ 78.6 (Δδ + 4.9 ppm) compared to those of the galactosyl residue found in lactose [12], indicating the occurrence of a 3-substitution at residue **B**. This substitution was verified by 2D ^{1}H-^{1}H ROESY NMR measurements (Fig. S6), displaying inter-residual cross-peaks between **C** H-1 and **B** H-3. Moreover, the chemical shift patterns of residues **B** and **C** are nearly identical to those in compound 3, suggesting the same structural element Combining all data, the structure of tetrasaccharide compound F5 was determined to be α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p*, as illustrated in Table 1.

2.3. High DP transglucosylation products of lactose

The reaction mixtures of Gtf180-ΔN and GtfA-ΔN incubated with lactose as acceptor substrate and sucrose as donor substrate were subjected to precipitation with 20% ethanol, followed by BioGel-P2 gel filtration (50 mL x 1.5 cm). The fraction size distribution in each pool was analyzed by MALDI-TOF-MS (Fig. S7). Subpool 1 contained structures of 3 and 4 hexose units (DP3-4; *m/z* 527 and 689). Subpool 2 consisted of structures of 5 and 6 hexose units (DP5-6; *m/z* 851 and 1013). Subpool 3 contained structures with 6 and 7 hexose units (DP6-7; *m/z* 1013 and 1175). Subpool 4 consisted of structures of 7 and 8 hexose units (DP7-8; *m/z* 1175 and 1337). Finally, subpool 5 contained structures of 8 and 9 hexose units (*m/z* 1337 and 1499). These fractions were subjected to 1D ^{1}H NMR analysis. The 1D ^{1}H NMR spectra of all fractions synthesized by Gtf180-ΔN revealed anomeric signals at δ 4.523 ppm, δ 4.510 ppm and δ 4.465 ppm (Fig. 2). These NMR resonances are indicative for a (−)β-D-Gal*p*-(1 → 4)- [12], confirming the occurrence of a galactosyl residue in the fragment with various degrees of polymerization higher than 4. The data shows that Gtf180-ΔN elongated lactose with glucose units up to DP9 (*m/z* 1661). However, in case of GtfA-ΔN, only the 1D ^{1}H NMR spectrum of fraction DP3-4 showed the anomeric signals at δ 4.523 ppm, δ 4.510 ppm and δ 4.465 ppm, indicative for a (−)β-D-Gal*p*-(1 → 4)-, fitting with the occurrence of F1-F5. In the subpools with higher DP, only α-D-glucan related signals were observed. These data suggest that GtfA-ΔN elongated lactose with only one or two glucosyl residues.

3. Discussion and conclusions

As previously reported for dextransucrases from *L. mesenteroides* and *W. confuse* [7,8] the glucansucrases from *L. reuteri* strains 121 and 180 also exhibit the ability to decorate

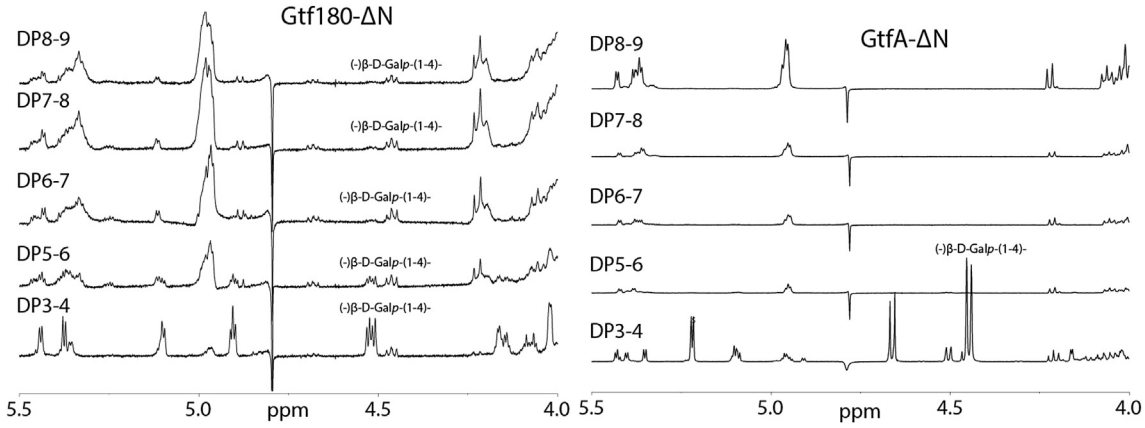


Fig. 2. 500-*MHz* 1D ^{1}H NMR spectra of BioGel-P2 fractions (DP3 to DP9) of (a) the incubation of Gtf180-ΔN with sucrose and lactose and (b) the incubation of GtfA-ΔN with sucrose and lactose, at the same conditions. Reaction conditions: sucrose and lactose at 0.5 M; 3 U mL^{-1} of enzyme; 24 h incubations at 37 °C and pH 4.7.

lactose with glucose. These dextransucrases apparently were only able to transfer a single glucose unit to lactose to form 2-α-D-glucopyranosyl-lactose (F2 in Table 1). At least five glucosylated lactose products with DP3 and DP4 were synthesized by GtfA-ΔN and Gtf180-ΔN and structurally characterized. In contrast to earlier findings that the linkage specificity of glucansucrases is conserved in oligosaccharide synthesis [13,14], new types of linkages were observed in the synthesized lactose glucosylation products. When using sucrose as donor and acceptor substrate, GtfA-ΔN synthesizes glucan with mainly (α1→4)/(α1→6) glucosidic linkages [10]; with lactose as acceptor substrate this enzyme introduced (α1→4) but also (α1→3) and (α1→2) glucosidic linkages. Similarly, Gtf180-ΔN produces an α-glucan with 69% (α1→6) and 31% (α1→3) linkages from sucrose [9], but with lactose as acceptor substrate it synthesized (α1→3) but also (α1→2) and (α1→4) glucosidic linkages. A most interesting finding was that GtfA-ΔN and Gtf180-ΔN synthesized the same set of DP3 and DP4 oligosaccharides from lactose as acceptor (F1-F5, Table 1). Only glucansucrase Gtf180-ΔN, however, produced larger oligosaccharides with a lactose core, most likely elongating the F2 structure further from its non-reducing end. These unexpected results reflect the regiospecificity of these glucansucrases in binding the lactose acceptor substrate in the active site. The structural features determining the product specificity of these Gtf enzymes acting on lactose as acceptor substrate remain to be elucidated.

In this study, the NMR spectral data of compound α-D-Glc*p*-(1 → 2)-[β-D-Gal*p*-(1 → 4)-]D-Glc*p* (F2) are consistent with data obtained from previous studies [7,8]. The full assignment of NMR spectra of two other trisaccharides (F1 and F3) and two tetrasaccharides (F4 and F5) are reported here for the first time. Lactose derivatives are interesting potential prebiotic compounds, especially those containing (α1→2)-linkages. These compounds are known to be highly resistant to the digestive enzymes in the human gut [15,16], and selectively stimulate the growth of health-beneficial microbiota [7,13]. The studied glucansucrases are able to elongate α-D-Glc*p*-(1 → 2)-[β-D-Gal*p*-(1 → 4)-]D-Glc*p* further with various types of linkages, such as α-D-Glc*p*-(1 → 4)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p* and α-D-Glc*p*-(1 → 3)-β-D-Gal*p*-(1 → 4)-[α-D-Glc*p*-(1 → 2)-]D-Glc*p*. Moreover, Gtf180-ΔN is able to produce glucosylated-lactose derivatives with a higher DP than 4. These results thus show that glucansucrases Gtf180-ΔN and GtfA-ΔN produce novel oligosaccharides (and putative prebiotic compounds) from cheap materials like lactose and sucrose. In our future research we will investigate the prebiotic properties of these glucosylated-lactose derivatives.

4. Experimental

4.1. Glucansucrase enzymes

Escherichia coli BL21 (DE3) (Invitrogen) carrying plasmid pET15b with the *gtf180* and *gtfA* genes from *Lactobacillus reuteri* strains 180 and 121 was used for expression of the N-terminally truncated glucansucrase enzymes (Gtf180-ΔN and GtfA-ΔN). The expression and purification of these glucansucrases have been described previously [17].

4.2. Transglucosylation reaction

The total activity of Gtf180-ΔN or GtfA-ΔN was measured as initial rates by methods described previously by Van Geel-Schutten et al. [18] The products of the transglucosylation reaction were prepared by incubating a mixture of 0.5 M sucrose (donor) and 0.5 M lactose (acceptor) with 3 U mL^{-1} glucansucrase at 37 °C in 50 mM sodium acetate buffer with 0.1 mM $CaCl_2$ at pH 4.7. The reaction was stopped after 24 h of incubation by heating at 100 °C for 10 min, followed by 400 times dilution of the inactivated sample with DMSO 95% and analyzed by High-pH anion-exchange chromatography (HPAEC-PAD).

4.3. Isolation and purification of oligosaccharide products

The reactions were carried out in a volume of 100 mL with the conditions described in section 4.2. Afterwards the reaction mixtures were mixed with two volumes of cold ethanol 20% and stored at 4 °C overnight to precipitate the polysaccharides. After centrifugation at 10,000 g for 10 min, the supernatant was applied to a rotatory vacuum evaporator to remove ethanol. The aqueous fraction was then absorbed onto a CarboGraph SPE column (Alltech, Breda, The Netherlands) using acetonitrile: water = 1:3 as eluent, followed by evaporation of acetonitrile under an N_2 stream before being freeze-dried. This was followed by fractionation HPAEC on a Dionex ICS-5000 workstation (Dionex, Amsterdam, the Netherlands), equipped with a CarboPac PA-1 column (250 × 9 mm; Dionex) and an ED40 pulsed amperometric detector (PAD). The gradient used for this fractionation is described in 4.4. The collected fractions were neutralized by acetic acid 20% and then desalted using a CarboGraph SPE column as described earlier.

4.4. HPAEC-PAD

The profiles of the oligosaccharides products were analyzed by HPAEC-PAD on a Dionex ICS-3000 work station (Dionex, Amsterdam, the Netherlands) equipped with an ICS-3000 pulse amperometric detection (PAD) system and a CarboPac PA-1 column (250 × 4 mm; Dionex). The analytical separation was performed at a flow rate of 1.0 mL min^{-1} using a complex gradient of effluents A (100 mM NaOH); B (600 mM NaOAc in 100 mM NaOH); C (Milli-Q water); and D: 50 mM NaOAc. The gradient started with 10% A, 85% C, and 5% D in 25 min—40% A, 10% C, and 50% D, followed by a 35-min gradient to 75% A, 25% B, directly followed by 5 min washing with 100% B and reconditioning for 7 min with 10% A, 85% B, and 5% D. External standards of lactose, glucose, fructose were used to calibrate for the corresponding sugars. For the determination of glucosylated lactose compounds with a degree of polymerization (DP) of 3, maltotriose was used as external standard.

4.5. MALDI-TOF mass spectrometry

Molecular mass of the compounds in the reaction mixture was determined by MALDI-TOF mass spectrometry on an Axima™ Performance mass spectrometer (Shimadzu Kratos Inc., Manchester, UK), equipped with a nitrogen laser (337 nm, 3 ns pulse width). Ion-gate cut-off was set to *m/z 200*and sampling resolution was software-optimized for *m/z* 1500. Samples were prepared by mixing 1 μL with 1 μL aqueous 10% 2,5-dihydroxybenzoic as matrix solution.

4.6. NMR spectroscopy

The structures of oligosaccharides of interest were elucidated by 1D and 2D ^{1}H NMR, and 2D^{13}C NMR. A Varian Inova 500 Spectrometer and 600 Spectrometer (NMR center, University of Groningen) were used at probe temperatures of 300 K with acetone as internal standard (chemical shift of δ 2.225). The aliquot samples were exchanged twice with 600 μL of $99.9_{\%atom}$ D_2O (Cambridge Isotope Laboratories, Inc., Andover, MA) by freeze-drying, and then dissolved in 0.65 mL D_2O, containing internal acetone. In the 1D ^{1}H NMR experiments, the data was recorded at 8 k complex data points, and the HOD signal was suppressed using a WET1D pulse. In the 2D ^{1}H-^{1}H NMR COSY experiments, data was recorded at 4000 Hz for both directions at 4k complex data points in 256 increments. 2D ^{1}H-^{1}H NMR TOCSY data were recorded with 4000 Hz at 30, 60, 100 spinlock times in 200 increments. In the 2D ^{1}H-^{1}H NMR ROESY, spectra were recorded with 4800 Hz at a mixing time of 300 ms in 256 increments of 4000 complex data points. MestReNova 5.9 (Mestrelabs Research SL, Santiago de Compostela, Spain) was used to process NMR spectra, using Whittaker Smoother baseline correction.

Acknowledgments

This work was financially supported by The Campus Fryslân, FrieslandCampina and The University of Groningen.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.carres.2017.07.002.

References

[1] B.I. Cantarel, P.M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, B. Henrissat, Nucleic Acids Res. 37 (2009) 233—238, http://dx.doi.org/10.1093/nar/gkn663.
[2] H. Leemhuis, T. Pijning, J.M. Dobruchowska, S.S. Leeuwen van, S. Kralj, B.W. Dijkstra, L. Dijkhuizen, J. Biotechnol. 163 (2013) 250—272, http://dx.doi.org/10.1016/j.jbiotec.2012.06.037.
[3] M. Balakrishnan, R.S. Simmonds, J.R. Tagg, Aust. Dent. J. 45 (2000) 235—245, http://dx.doi.org/10.1111/j.1834-7819.2000.tb00257.x.
[4] T. Devlamynck, E.M. Poele te, X. Meng, S.S. van Leeuwen, L. Dijkhuizen, Appl. Microbiol. Biotechnol. (2016) 7529—7539, http://dx.doi.org/10.1007/s00253-016-7476-x.
[5] M. Dols, M.R. Simeon, R.M. Willemot, M.R. Vignon, P.F. Monsan, Carbohydr. Res. 305 (1997) 549—559, http://dx.doi.org/10.1016/S0008-6215(97)10063-5.
[6] V. Monchois, R.M. Willemot, P. Monsan, FEMS Microbiol. Rev. 23 (1999) 131—151, http://dx.doi.org/10.1016/S0168-6445(98)00041-2.
[7] M. Díez-Municio, A. Montilla, M.L. Jimeno, N. Corzo, A. Olano, F.J. Moreno, J. Agric. Food Chem. 60 (2012) 1945—1953, http://dx.doi.org/10.1021/jf204956v.
[8] Q. Shi, M. Juvonen, Y. Hou, I. Kajala, A. Nyyssölä, N.H. Maina, H. Maaheimo, L. Virkki, M. Tenkanen, Food Chem. 190 (2016) 226—236, http://dx.doi.org/10.1016/j.foodchem.2015.05.090.
[9] S.S. van Leeuwen, S. Kralj, I.H. van Geel-Schutten, G.J. Gerwig, L. Dijkhuizen, J.P. Kamerling, Carbohydr. Res. 343 (2008) 1237—1250, http://dx.doi.org/10.1016/j.carres.2008.01.042.
[10] S.S. van Leeuwen, S. Kralj, I.H. van Geel-Schutten, G.J. Gerwig, L. Dijkhuizen, J.P. Kamerling, Carbohydr. Res. 343(2008) 1251—1265. doi: 10.1016/j.carres.2008.01.044.
[11] S.S. van Leeuwen, B.R. Leeflang, G.J. Gerwig, J.P. Kamerling, Carbohydr. Res. 343 (2008) 1114—1119, http://dx.doi.org/10.1016/j.carres.2008.01.043.
[12] S.S. van Leeuwen, B.J.H. Kuipers, L. Dijkhuizen, J.P. Kamerling, Carbohydr. Res. 400 (2014) 54—58, http://dx.doi.org/10.1016/j.carres.2014.08.011.
[13] M. Remaud-Simeon, R.M. Willemot, P. Sarçabal, G. Potocki De Montalk, P. Monsan, J. Mol. Catal. - B Enzym (2000) 117—128, http://dx.doi.org/10.1016/S1381-1177(00)00119-3.
[14] V. Monchois, A. Reverte, M. Remaud-simeon, P. Monsan, R. Willemot, Appl. Environ. Microbiol. 64 (1998) 1644—1649.
[15] P. Valette, V. Pelenc, Z. Djouzi, C. Andrieus, F. Paul, P. Monsan, O. Szylit, J. Sci. Food Agric. 62 (2) (1993) 121—127, http://dx.doi.org/10.1002/jsfa.2740620204.
[16] T. Nakada, T. Nishimoto, H. Chaen, S. Fukuda, Oligosaccharides Food Agric. 849 (2003) 104—117, http://dx.doi.org/10.1021/bk-2003-0849.ch009.
[17] S. Kralj, G.H. van Geel-Schutten, M.M.G. Dondorff, S. Kirsanovs, M.J.E.C. Maarel van der, L. Dijkhuizen, Microbiology 150 (2004) 3681—3690, http://dx.doi.org/10.1099/mic.0.27321-0.
[18] G.H. Van Geel-Schutten, E.J. Faber, E. Smit, K. Bonting, M.R. Smith, B. Brink ten, J.P. Kamerling, J.F.G. Vliegenthart, L. Dijkhuizen, Appl. Environ. Microbiol. 65 (1999) 3008—3014.